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The Relationship Between NO, ADMA and Homocysteine in Endotoxin-Mediated Inflammation in HUVEC Cultures

[HUVEC Kültürlerinde Endotoksin-Aracılı İnflamasyonda NO, ADMA ve Homosistein Arasındaki İlişki]

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ABSTRACT

Objectıve: The aim of our study was to investigate the relationship between Nitric Oxide, ADMA, homocysteine in endotoxin-induced human umblical vein endothelial cell cultures.

Methods: For this reason two groups are formed (n=12). Control group consists of HUVEC cultures without any treatment. LPS group is treated with 10µg/mL endotoxin (E.coli 0111: B4).The measurement of NO levels is perfomed by spectrophotometric measurement of nitrite and nitrate. ADMA and Homocysteine levels are measured using HPLC with fluorescence detection.

Results: The results of our study showed that there was a significant increase of NO, ADMA and Homocysteine in endotoxemia ($p<0.05$).

Conclusıon: In our inflammation model, both Arginine-ADMA and Arginine-NO pathways are found to be activated. In this model, the NOS isoform catalysed NO synthesis is iNOS. We speculate ADMA, which was formed as a result of LPS stimulation, was not sufficient to inhibit iNOS and it also caused eNOS to uncouple and contributed to peroxynitrite formation, thus lead to a dramatic increase in nitrate/nitrite levels. Furthermore, increased oxidative stress caused by elevated homocysteine levels might have contributed to increased NOx levels via iNOS activation and ADMA accumulation. As a result, endothelial dyfunction that may arise from the relationship between NO-ADMA-Homocysteine might provide a different point of view to atherosclerotic diseases.

Key Words: nitric oxide, ADMA, homocysteine, HUVEC, endotoxemia

Conflict of Interest: Authors have no conflict of interests.

ÖZET

Amaç: Çalışmamızda, endotoksinle uyarılmış insan umblikal ven endotel hücresi (HUVEC) kültürleri mediumlarında Nitrik Oksit (NO), ADMA ve Homosistein arasındaki ilişkiyi araştırmayı amaçladık.

Yöntemler: Bu amaçla, iki grup oluşturduk (n=12). Kontrol grubu hiçbir uygulama yapılmamış HUVEC kültürlerini, LPS grubu ise 10µg/mL endotoksin (E.coli 0111: B4) ile muamele edilmiş HUVEC kültürlerini kapsamaktadır. NO düzeylerinin ölçümü nitrit ve nitratın spektrofotometrik ölçümüyle yapıldı. ADMA ve Homosistein düzeylerinin ölçümleri ise HPLC ile flüoresans deteksiyon kullanılarak gerçekleştirildi.

Bulgular: Sonuçlarımız, endotoksemik koşullarda NO, ADMA ve Homosistein düzeylerinde artış olduğunu gösterdi ($p<0.05$).

Sonuç: İnflamasyon, aterosklerotik hastalıklar için karakteristiktir ve endotel disfonksiyonu aterogenezin tüm aşamalarında rol oynamaktadır. İnflamasyon modelimizde, Arjinin-ADMA ve Arjinin-NO yollarının ikisinin de aktive olduğu bulunmuştur. Bu modelde, NO sentezini katalizleyen NOS izoformu iNOS'tur. LPS uyarılması sonucu oluşan ADMA'nın iNOS'u inhibe etmeye yetmediğini ve aynı zamanda eNOS'un ayrılmasına neden olarak peroksinitrit oluşturmaya yönlendirdiğini ve bu şekilde nitrat/nitrit seviyelerinde dramatik bir artış görüldüğünü düşünüyoruz. Bunun yanında, homosistein düzeylerindeki artışın neden olduğu oksidatif stres de iNOS aktivasyonuna ve ADMA birikimine neden olmak yoluyla NOx düzeylerinde artışa neden olmuş olabilir. Sonuç olarak, NO-ADMA-Homosistein arasındaki ilişkiden ortaya çıkan endotel disfonksiyonun aterosklerotik hastalıklar için farklı bir bakış açısı sağlayabileceği kanısındayız.

Anahtar Kelimeler: nitrik oksit, ADMA, homosistein, HUVEC, endotoksemi

Çıkar Çatışması: Yazarların çıkar çatışması yoktur.

Introduction

Endothelial cells participate in inflammation with specific adaptive response [1]. The adaptive response of endothelial cells to stres includes formation of reactive oxygen species (ROS) [2] and may lead to the upregulation of nitric oxide (NO) production [1].

Nitric oxide (NO) synthesis via the enzymatic oxidation of guanidino nitrogen of one terminal of the amino acid L-arginine (L-arg) is catalysed by the enzyme nitric oxide synthase (NOS) [3] plays an important role in conserving vascular structure and function [1-5]. While small amount of NO, synthesized by endothelial NOS (eNOS) provides physiological events like vasodilatation, large amount of NO is formed by inducible NOS (iNOS) during an inflammation state triggered by cytokines and endotoxin, and effects endothelial cell function negatively, leading to endothelial dysfunction [1, 3, 6]. Endothelial dysfunction plays an important role in all stages of atherosclerotic diseases [5, 7].

Asymmetric dimethylarginine (ADMA) is a L-arginine analog that functions as an endogenous inhibitor of all NOS isoforms and causes decreased NO levels [6, 8, 9]. ADMA is involved in the pathophysiology of human vascular diseases and considered to be not only a marker of endothelial dysfunction but also an important cardiovascular risk factor [6, 10].

Elevated levels of homocysteine (Hcy) has been suggested to participate in endothelial dysfunction and increased risk of vascular diseases [11-14]. Hcy is synthesised from the amino acid methionine by a demethylation reaction [11,12].

Some researchers suggest that L-arginine may function as a methyl group acceptor during the demethylation of methionine to homocysteine, resulting in increased formation of ADMA [13]. On the other hand, some researchers claim that increased plasma levels of asymmetric dimethylarginine, which is found statistically unsignificant, in patients with high plasma total homocysteine levels may be explained by renal dysfunction [14].

As it is clearly seen from the literature, NO, ADMA and Hcy levels are not evaluated together and there are conflicts between the results. Furthermore, most of the studies are based on loading prosedures like methionine loading or ADMA administration to cultures. We aimed to investigate the natural relationship between these three parameters in an in vitro model, using primary HUVEC cultures, in an inflammatory state, which is known to participate in all stages of endothelial dysfunction and atherosclerosis, using lipopolisaccharide (LPS) stimulation.

Materials and Methods

Materials

Medium 199 and HEPES are purchased from Gibco (CA, USA). Feutal bovine serum (FBS), Penicillin, Streptomycin and Collagenase Type II are purchased from PAA: The cell culture company (Pasching, Austria). Gelatin, lipopolisaccaride (LPS E.coli 0111:B4) and all chemicals needed for ADMA measurement are purchased from Sigma-Aldrich Co. (MO, USA). Nitrate/ Nitrite Colorometric Assay Kit is purchased from Cayman Chemical (Michigan, USA) and homocysteine kit from Immuchrom (Heppenheim, Germany).

Cell Culture and Treatment

Human umblical cords were obtained from deliveries with cesarean section to minimize a risk of contamination and kept in Krebs-Henseleit solution until isolation which is performed on the same day. Endothelial cells were isolated from human umbilical cords by digestion of the interior of the umbilical vein with collagenase type II as previously described. [15] Cells were plated in 1% gelatin coated plates. The medium, M199, contains 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 0.135% $NaHCO_3$, 15mM HEPES and 2 mM glutamine. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. Medium was changed 24 h after plating and subsequently every 2 days until confluence. Experiment was performed on confluent primary cultures. After the HUVECs reached confluence, 10µg/mL LPS (E.coli 0111:B4), which was known to be a sufficient dose for induction [16], was added to the cell culture media of the LPS group. Cells were incubated for 24 h at 37°C in humidified 5% CO_2 atmosphere. After the incubation period, medium was taken and stored at -20°C.

Measurement of Nitrite/Nitrate (NOx) levels

The measurement of NOx levels was perfomed by spectrophotometric measurement of end products, nitrite and nitrate [17], using a commercially available kit (Cayman Chemical, Michigan, USA).

Measurement of Hcy levels

The measurement of Hcy was implemented by using HPLC with florescent detection (Ex=385nm ; Em=515nm) [18], using Immuchrom (Heppenheim, Germany) homocysteine kit.

Measurement of ADMA levels

ADMA levels were measured as previously described by Chen et al. [19] using HPLC with florescent detection (Ex=338nm ; Em=425nm).

Statistic Analysis

Results are expressed as means ± standard deviation. The data were analysed by Mann-Whitney U test. The statistical significance level was chosen as $p<0,05$.

Results

The medium nitrate-nitrite, ADMA and Hcy levels are shown in the Table.

Table. Medium NOx, Hcy and ADMA levels of the groups

	NOx (µmol/L)	Hcy (µmol/L)	ADMA (µmol/L)
Control group (n=12)	3.99 ± 0.84	2.98 ± 0.19	3.46 ± 0.48
LPS group (n=12)	11.98 ± 0.66 [a]	3.92 ± 0.67 [b]	4.77 ± 0.42 [b]

Results were expressed as mean ± standard deviation
[a] $p<0.01$, compared to the corresponding value of control group
[b] $p<0.05$, compared to the corresponding value of control group

NOx levels

LPS administration significantly increased nitrate-nitrite levels ($p<0,01$). Elevation of NOx levels was considered as successful demonstration of an experimental endotoxemia model in HUVEC cultures.

Homocysteine levels

Hcy levels were found to be signigicantly increased in LPS group compared to control group ($p<0.05$).

ADMA levels

Endotoxin stimulation significantly increased ADMA levels of LPS group compared to control group ($p<0.05$).

Discussion

Endothelial cells undergo some biochemical changes as a response to stress at *in vivo* as well as in vitro conditions [1,2]. These changes include increase of nitric oxide (NO) production [1] and free radical formation [2].

Previous studies proved that stress [3], LPS and cytokine stimulation [20] leads to elevation of NO levels by iNOS activation. In our study, we measured nitrate/nitrite levels, which are known to be end products of NO [17] and established a statictically significant increase in LPS group compared to control group. We considered this data as a marker of successful stimulation by endotoxin and demonstration of an inflammation model.

ADMA, which is an arginine analogue, is a newly recognized marker and a risk factor for cardiovascular diseases [6, 10]. Mittermayer et al. [21] could not find significant difference in plasma ADMA levels after endotoxemia compared to healthy individuals. In contrast, Balabanlı et al. [22] reported that plasma ADMA levels of guinea pigs increased, due to activation of arginine-ADMA pathway, after endotoxin administration. We compared ADMA levels of the two groups after the 24 hours incubation period with LPS and detected a statistically significant elevation of ADMA levels.

In the inflammation model we generated using LPS stimulation, both Arginine-ADMA and Arginine-NO pathways are found to be activated. On first sight, increase in both ADMA and nitrate/nitrite levels seem to be a contrary finding as it is known that ADMA is an endogenous inhibitor of NOS and increased levels of ADMA would decrease NO, thereby nitrate/nitrite levels [6, 8, 9]. However, in this model, it is obvious that NOS isoform catalysed NO synthesis is iNOS as endotoxin stimulates iNOS [20]. Bestermann [23] and Cardounel et al. [24] reported that ADMA uncouples eNOS and causes a shift from NO to peroxynitrite production. In a study, it is claimed that while it is known that ADMA is a strong inhibitor of eNOS, it weakly inhibited NO production by iNOS in LPS/cytokine stimulated mouse lung epithelial cells [6]. Also it is reported that ADMA increases iNOS [23]. Our data supports these finding regarding to the increase of both ADMA and nitrate/nitrite levels. In our study, we speculate that because of iNOS activation lead by LPS stimulation plus ADMA accumulation and also because of the generation of peroxynitrite, total nitrate/nitrite levels are found elevated.

Another parameter we measured in HUVEC cultures is homocysteine (Hcy), a clinically used marker and risk factor for cardiovascular diseases [25]. Our results showed that homocysteine levels of the culture medium significantly increased, like nitrite/nitrate and ADMA levels, after incubation with LPS. There are conflicting data about the relationship between NO and homocysteine. Upchurch et al. [26] reported that homocysteine stimulates NO production by stimulating eNOS. According to Woo et al. [27], homocysteine stimulates iNOS expression in macrophages. In contrast, Zhang et al. [28] incubated endothelial cells with homocysteine and their daha showed that homocysteine did not effect expession of eNOS and it did not induce iNOS expression, but stimulated formation of free radicals. In another study that compared ADMA production after incubation with homocysteine, the researchers reported that ADMA levels are elevated, due to dimethylamine dimethylaminohydrolase (DDAH) activity decrease and NO levels are diminished, due to NOS inhibition by ADMA [29]. Our results show similarities with the study made by Tyagi et al. [30], which the researchers incubated endothelial cells with homocysteine and reported that Hcy induced iNOS, and there was significant accumulation of ADMA.

In vitro studies made to investigate NO-homocysteine relationship or NO-ADMA-Homocysteine relationship is mainly based on incubation with homocysteine. However, we measured the natural ADMA, Hcy and nitrite/nitrate, thereby NO values after an inflammatory stimulation. The differences might be due to experimental design differences and as all of these molecules form endogenously in human, i.e. not

taken into the body exogenously, we think that our data implies a natural relationship on an inflammatory state. It is impossible to compare and evaluate our study with another research completely because there is no study investigated NO, ADMA and Hcy parameters together in an endotoxin mediated inflammatory state.

In conclusion, endothelial dysfunction arised from NO-ADMA-Homocysteine relationship in an inflammatory state, like endotoxin-mediated inflammation, might provide a different point of view to initiation and progression of atherosclerotic diseases. However, further studies like Western Blot analysis of eNOS and iNOS proteins should be made.

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Conflict of Interest: Authors have no conflict of interests.

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